N-Cadherin Promotes Motility in Human Breast Cancer Cells Regardless of their E-Cadherin Expression

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Abstract. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell–cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a nonepithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to downregulate E-cadherin expression and to contribute to a scattered phenotype. In this study, we explored the possibility that expression of nonepithelial cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motility or invasion; that N-cadherin expression correlates both with invasion and motility, and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin–positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in noninvasive, E-cadherin–positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; that N-cadherin–dependent motility may be mediated by FGF receptor signaling; and that cadherin-11 promotes epithelial cell motility in a manner similar to N-cadherin.

Key words: N-cadherin • E-cadherin • breast cancer • motility • fibroblast growth factor receptor

CADHERINS constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell–cell adhesion and play an important role in the maintenance of normal tissue architecture. The cadherin intracellular domain interacts with several proteins, collectively called catenins, that link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). This linkage is required for full cadherin adhesive activity. Either β-catenin or plakoglobin binds directly to the cadherin and to α-catenin, whereas α-catenin links directly and indirectly to actin (Aberle et al., 1994; Nagafuchi et al., 1994; Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998). Their ability to simultaneously self-associate and link to the actin cytoskeleton enables cadherins to mediate both the cell recognition required for cell sorting and the strong cell–cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β-catenin in Src transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). In addition, $p120^{ctn}$, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Aono et al., 1999; Ohkubo and Ozawa, 1999).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen and Öbrink, 1991). In addition, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991). Moreover, E-cadherin expression is downregulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991; Bringuier et al., 1993; Dorudi et al., 1993; Mayer et al., 1993; Oka et al., 1993; Umbas et al., 1994).

It has been suggested that alterations in cadherin function may be a critical step in the development of breast cancers. A survey of 18 cell lines derived from breast carci-

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nomas showed that ten lines failed to express detectable levels of E-cadherin, and two other lines failed to express α-catenin (Pierceall et al., 1995). Other studies have identified breast tumor cell lines with mutations in the E-cadherin gene (Berx et al., 1995), or with changes in the levels of expression or in the phosphorylation state of β-catenin or plakoglobin (Sommers et al., 1994). Surveys of breast cancer tissue make an equally compelling case for the involvement of E-cadherin in the formation or progression of breast tumors, and clinical studies have shown that loss of E-cadherin correlates with metastatic disease and poor prognosis (Gamello et al., 1993; Moll et al., 1993; Oka et al., 1993; Rasbridge et al., 1993; Berx et al., 1996; Guriec et al., 1996).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. For example, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin–negative breast or prostate cell lines with mouse E-cadherin resulted in cells that were less invasive in in vitro assays (Frixen et al., 1991; Luo et al., 1999). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype, thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991).

Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin–negative cell lines were shown to be noninvasive (Sommers et al., 1991). These authors suggested that in order for E-cadherin–negative cells to be invasive, they must also express vimentin.

In another study, Sommers et al. (1994) showed that transfection of E-cadherin into the invasive breast cancer cell lines, BT549 and HS578t, altered neither the morphology nor the invasive behavior of these cells. These authors speculated that the transfected E-cadherin may not be fully functional in these cells, due to altered posttranslational modification of the cadherin-associated proteins β-catenin, α-catenin, or plakoglobin.

It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion, and suggested that invasion was potentiated by N-cadherin–mediated interactions between the breast cancer cells and stromal cells. A study conducted in our laboratory suggested that N-cadherin may play a more direct role in the process of invasion and may actually promote invasion by inducing a scattered phenotype when expressed by oral squamous cell carcinoma-derived cells (Islam et al., 1996). In this study, forced expression of N-cadherin resulted in downregulation of endogenous E- and P-cadherins, making it impossible to separate the motility-promoting effects of N-cadherin from the motility-suppressing activity of E-cadherin. In contrast, it has been suggested that N-cadherin promotes contact inhibition in normal skeletal muscle myoblasts and, in so doing, inhibits migration upon contact, but does not suppress motility in subconfluent cells (Huttenlocher et al., 1998).

Thus, the information in the literature concerning the role cadherins may play in tumor cell invasion is inconclusive and even contradictory, prompting us to revisit the question using new reagents generated by our laboratory to examine both previously studied and newly derived breast cancer cell lines. The data presented in this paper indicate: decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; N-cadherin expression correlates both with invasion and motility in breast cancer cells, and likely plays a direct role in promoting motility; forced expression of E-cadherin in invasive, N-cadherin–positive cells does not reduce their motility or invasive capacity; forced expression of N-cadherin in noninvasive, E-cadherin–positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; the data suggest that N-cadherin-mediated cell motility may be stimulated by FGF receptor signaling; and other cadherins, such as cadherin-11, may promote motility in epithelial cells in a manner similar to N-cadherin.

Materials and Methods

Cells

Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and maintained in DME with 10% FBS (SKBr3, MDA-MB-435, MDA-MB-436, BT-549, and Hs578t) or MEM with 10% FBS (MDA-MB-453 and BT-20). The cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Mary J.C. Hendrix (University of Iowa, Iowa City, IA) and maintained in DME with 10% FBS. The cell lines SUM 159PT and SUM 149 were kindly provided by Dr. Steve Ethier and generated by the University of Michigan Human Breast Cell/Tissue Bank and Data Base. They were maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM 1315 was obtained from the same source and maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and EGF (10 ng/ml). HT1080 cells were obtained from ATCC and maintained in DME with 10% FBS.

Transfections

To transfect MDA-MB-435 with E-cadherin, the calcium phosphate transfection kit (Stratagene) was used, according to manufacturer's protocol. For electroporations (BT-20 cells), 10^6 cells were washed with PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$) supplemented with 2 mM ATP and 5 mM glutathione. After a 5 min incubation on ice, the cells were electroporated at 500 μF and 380 V in a Bio-Rad gene pulser. Cells were immediately plated in a 100-mm dish in complete medium. Floating cells were removed and fresh medium was added 24 h after electroporation; puromycin was added to the culture for selection of clones 48 h after electroporation.

Clones and Vectors

For transfection of N-cadherin, a restriction fragment containing nucleotides 442–3362 (GenBank/EMBL/DDBJ accession number S42303; a kind gift of Dr. Avri Ben-Ze'ev, The Weizmann Institute of Science, Israel) was ligated into the expression vector pLK-pac (Islam et al., 1996). The E-cadherin construct has been described previously (Lewis et al., 1997). The human cadherin-11 cDNA was provided by Drs. S. Takashita and A. Kudo (Tokyo Institute of Technology, Japan; accession number D21254; Okazaki et al., 1994).

Antibodies and Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987), and mouse mAbs against E-cadherin

(HECD1; a kind gift of Dr. Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995, Sacco et al., 1995), have been described previously. The mouse mAb against β-catenin (6E3) was made as described by Johnson et al. (1993). The mouse mAbs against cadherin-11 were kindly provided by Dr. Marion Bussemakers (University Hospital Nijmegen, The Netherlands). The diacylglycerol lipase inhibitor, RHC80267, was purchased from BIOMOL.

Extraction of Cells

Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 ml/75 cm^2 flask 10 mM Tris acetate, pH 8.0, containing 0.5% NP-40 (BDH Chemicals Ltd.), 1 mM EDTA, and 2 mM PMSF. The cells were scraped, followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 *g* for 10 min at 4°C. Cell extracts were resolved on 7% SDS-PAGE as described (Lewis et al., 1994), transferred electrophoretically to nitrocellulose, and immunoblotted as described (Wheelock et al., 1987) using primary antibodies followed by ECL, according to the manufacturer's protocol (Pierce Chemical Co.). For the purpose of loading equal amounts of protein onto SDS-PAGE, quantification was done using the BioRad protein assay reagent according to the manufacturer's protocol.

Immunofluorescence and Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), washed three times with PBS, and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed three times with PBS, and exposed to species-specific antibodies conjugated to FITC or rhodamine for 1 h. Cells were viewed using a Zeiss Axiophot microscope equipped with the appropriate filters, and photographed using Kodak T-MAX 3200 film. Living cells were viewed using a Zeiss Axiovert microscope and photographed using Kodak T-MAX 400 film.

In Vitro Invasion Assays and Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of noncoated polyethylene teraphthalate (PET) membranes (6-well insert, pore size 8 mm; Becton Dickinson). For in vitro invasion assays, 3×10^4 cells were plated in the top chamber of Matrigel-coated PET membranes (24-well insert, pore size 8 mm; Becton Dickinson). In motility and invasion assays, 3T3 conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade). Cells in ten random fields of view at 100× were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data were represented as the average of the three independent experiments with the SD of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone. To inhibit FGF receptor signaling, cells were treated with RHC80267 (which inhibits the activity of diacylglycerol lipase) at a concentration of 10–40 μg/ml 3T3 conditioned culture medium during the 24 h of the assay.

Results

Expression of Cadherins by Breast Cancer Cells

E-cadherin has been termed a tumor suppressor, mainly because cells derived from E-cadherin–negative epithelial tumors tend to be invasive, whereas cells derived from E-cadherin–positive tumors tend not to be. In the case of cells derived from breast carcinomas, the majority of E-cadherin–negative cells are invasive (Sommers et al., 1991, 1994; Pierceall et al., 1995). However, an increasing number of exceptions to this rule are becoming evident. Our laboratory has recently shown that expression of an inappropriate cadherin by an oral squamous carcinoma cell line influences expression of E-cadherin and the cellular phenotype (Islam et al., 1996). This observation led us to hypothesize that the invasiveness of some breast cancer cells may be due to an increase in the expression of an inappropriate cadherin, possibly N-cadherin, rather than to a decrease in the expression of E-cadherin. To test this hypothesis, we surveyed a large number of cell lines, many of which had been characterized previously, for expression of E- and N-cadherin. The data, which are summarized in Table I, supported our notion that invasiveness is correlated with N-cadherin expression, rather than lack of E-cadherin expression.

Table I. Cadherin Expression in Breast Carcinoma Cell Lines

Cell line	E-cadherin	N-cadherin	P-cadherin	Cadherin-11	β-Catenin	Motility
MCF-7	+*‡	−*	−*	−*	+*‖¶	No*‡
BT-20	+*	−*	+*	−*	+*	No*
SUM149	+*	−*	+*	−*	+*	No*
SKBr3	−*‡	−*	−*	−*	−*‖¶	No*‡
MDA-MB-453	−*‡	−*	−*	−*	−*‖¶	No‡
SUM1315	−*	−*	+*	±*	+*	No*
MDA-MB-435	−*‡	+*	−*	−*	+*¶	Yes§
MDA-MB-436	−*‡	+*	−*	−*	+*‖¶	Yes‡
BT549	−*‡	+*	+*	−*	+*‖¶	Yes‡
Hs578t	−*‡	+*	−*	−*	+*‖¶	Yes*‡
SUM159PT	−*	+*	−*	−*	+*	Yes*
MDA-MB-231	−*	−*	−*	+*†	+*	Yes*†

*Current study; ‡Sommers et al., 1991; §Frixen et al., 1991; ‖Sommers et al., 1994; ¶Pierceall et al., 1995; †Pishvaian et al., 1999.

Fig. 1 is an immunoblot of extracts of the cell lines presented in Table I. Equal amounts of protein were loaded in each lane. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-, N-, or P-cadherin, cadherin-11, and β-catenin. Fig. 2 presents phase micrographs of the living cells to compare the

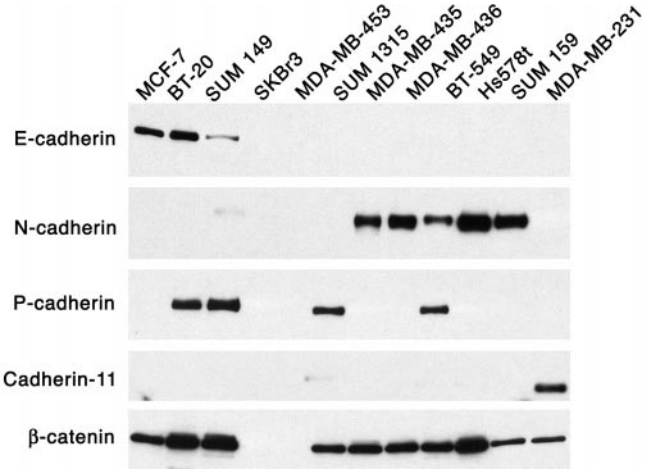

Figure 1. Cadherin and β-catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, BT-20, SUM 149, SKBr3, MDA-MB-453, SUM 1315, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM 159PT, or MDA-MB-231 were extracted with NP-40. 20 μg total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against E-cadherin (HECD1), N-cadherin, P-cadherin, cadherin-11, or β-catenin.

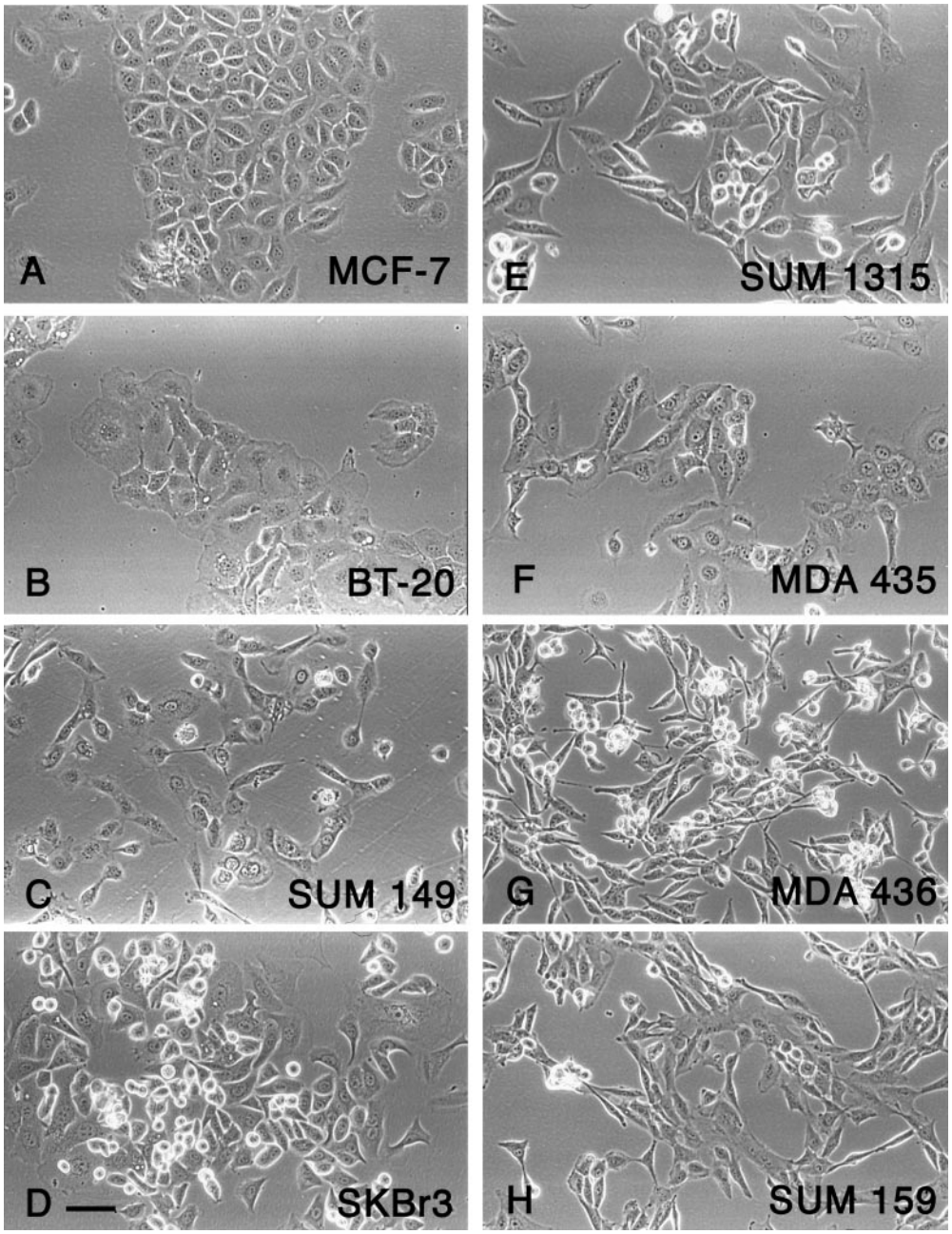

Figure 2. Morphological analysis of breast cancer cell lines. Living monolayers of MCF-7 (A), BT-20 (B), SUM 149 (C), SKBr3 (D), SUM 1315 (E), MDA-MB-435 (F), MDA-MB-436 (G), or SUM 159PT (H) cells were photographed using an inverted Zeiss microscope at 200×. Bar, 10 μm.

morphologies of breast cancer cells expressing the various members of the cadherin family. MCF-7 cells expressed E-cadherin, had low invasion rates, and presented an epithelial-like morphology. BT-20 cells expressed both E- and P-cadherin, had low invasion rates, and presented an epithelial-like morphology. In contrast, E-cadherin–negative cell lines did not present an epithelial morphology, but rather appeared as fibroblast-like cells with less obvious cell–cell interactions. Even the SUM149 cell line that expressed a small amount of E-cadherin, along with substantial amounts of P-cadherin, did not have the epithelial appearance typified by the MCF-7 and BT-20 cell lines. SUM1315 cells, which expressed P-cadherin, along with a small amount of cadherin-11, also had a fibroblastic appearance with minimal cell–cell interactions. However, these fibroblastic, N-cadherin–negative cell lines had low motility and invasion rates (Table I and Fig. 3). The N-cadherin–expressing cell lines all displayed a fibroblastic phenotype, as typified by MDA-MB-435, MDA-MB-436, and SUM159 (Fig. 2). Cell lines that did not express any cadherin, as typified by SKBr3, displayed a fibroblastic phenotype much like the N-cadherin–positive cells, however, they were less adhesive to the substratum than were cadherin-expressing cells. In addition, they tended to float in the medium upon reaching confluency and when undergoing mitosis.

A Role for N-Cadherin in Cell Motility

In this study, we hypothesized that the invasive behavior

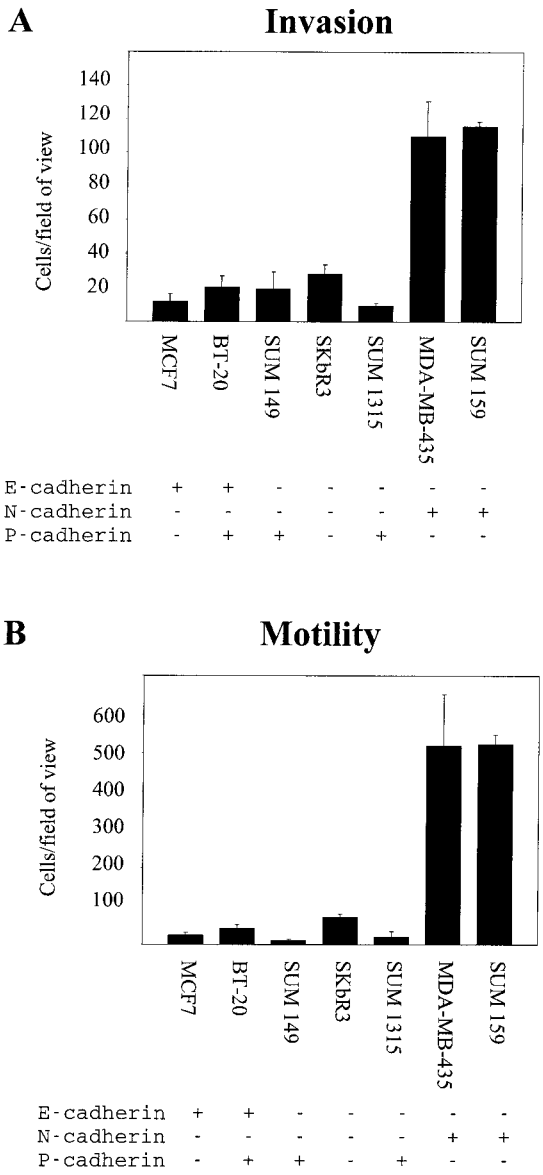

Figure 3. N-cadherin expression correlates with increased invasiveness and motility in breast carcinoma cell lines. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

of some breast cancer cell lines may be due to expression of N-cadherin, rather than to lack of expression of E-cadherin. To test this hypothesis, we performed invasion assays on Matrigel-coated membranes and motility assays on uncoated membranes. Fig. 3 presents data from representative cell lines. The N-cadherin–expressing cell lines, SUM159 and MDA-MB-435, were substantially more invasive and more motile than the E-cadherin–expressing line (MCF-7), the E/P-cadherin–expressing cell lines (BT-20 and SUM149), and the P-cadherin–expressing line (SUM 1315). The cell line that did not express any cadherins, SKBr3, was no more motile nor invasive than were the E-cadherin–expressing cell lines BT-20, MCF-7, and SUM 149. Together, these data suggest that, in these cells, N-cadherin acts to promote motility and invasion, rather than E-cadherin acting to suppress these activities.

Since the cell lines in this study were derived from separate tumors and, thus, are likely to be descendents of different cell types, we sought to manipulate expression of specific cadherins in representative cell lines to determine if the invasive phenotype was due to N-cadherin or to other cellular aspects. We chose two cell lines for these studies: BT-20, which expresses E- and P-cadherin and has a low rate of invasion, and MDA-MB-435, which expresses N-cadherin and is highly invasive. When BT-20 cells were transfected with N-cadherin (BT-20N), they expressed levels of N-cadherin that were comparable to MDA-MB-435; however, they did not undergo a morphological change (compare Fig. 2 B with Fig. 4 A), nor did they downregulate the expression of E-cadherin to any significant level. Fig. 4, B and C, show that E- and N-cadherin colocalized at cell–cell borders, suggesting that both cadherins are active at the cell surface. When equal amounts of protein from extracts of BT-20 and BT-20N cells were resolved by SDS-PAGE and immunoblotted for cadherin expression, it could be seen that the BT-20N cells slightly downregulated E-cadherin, that the two cell lines expressed equal levels of P-cadherin, and that the BT-20N cells expressed levels of N-cadherin that were comparable to the invasive N-cadherin–expressing cells depicted in Fig. 1. In addition, β-catenin coimmunoprecipitated equally well with either E- or N-cadherin in these cells (Fig. 4 E). BT-20 cells were unusual in that they expressed high levels of both E- and N-cadherin and, thus, were an ideal cell line in which to test the hypothesis that it is the expression of N-cadherin, not the lack of E-cadherin, that promotes cell motility and invasion in some breast cancer cells. As predicted, motility and invasion rates for BT-20N were five- to eightfold higher than the rates for nontransfected BT-20 cells (Fig. 5). Although BT-20N cells were not as motile as the N-cadherin–expressing MDA-MB-435 cells (Fig. 5 B), they were almost as invasive (Fig. 5 A).

E-Cadherin Does Not Suppress Motility in N-Cadherin–expressing MDA-MB-435 Cells

Since the BT-20N cells expressed high levels of E-cadherin, and were highly motile and invasive, we had good evidence that E-cadherin did not inhibit invasion in these cells and, thus, does not act as an invasion suppressor in all breast cancer cells. However, to further test this idea, we transfected N-cadherin–expressing MDA-MB-435 cells with E-cadherin (MDA-MB-435E) to see if E-cadherin would decrease the invasive nature of these cells. In this experiment, we sought to obtain clones that expressed high levels of E-cadherin, but still retained a significant level of N-cadherin. Fig. 6 D shows the levels of expression of E- and N-cadherin in several clones. Clone 2 was chosen for subsequent studies because it expressed the highest level of E-cadherin and, in addition, showed a two- to threefold reduction in N-cadherin expression, compared

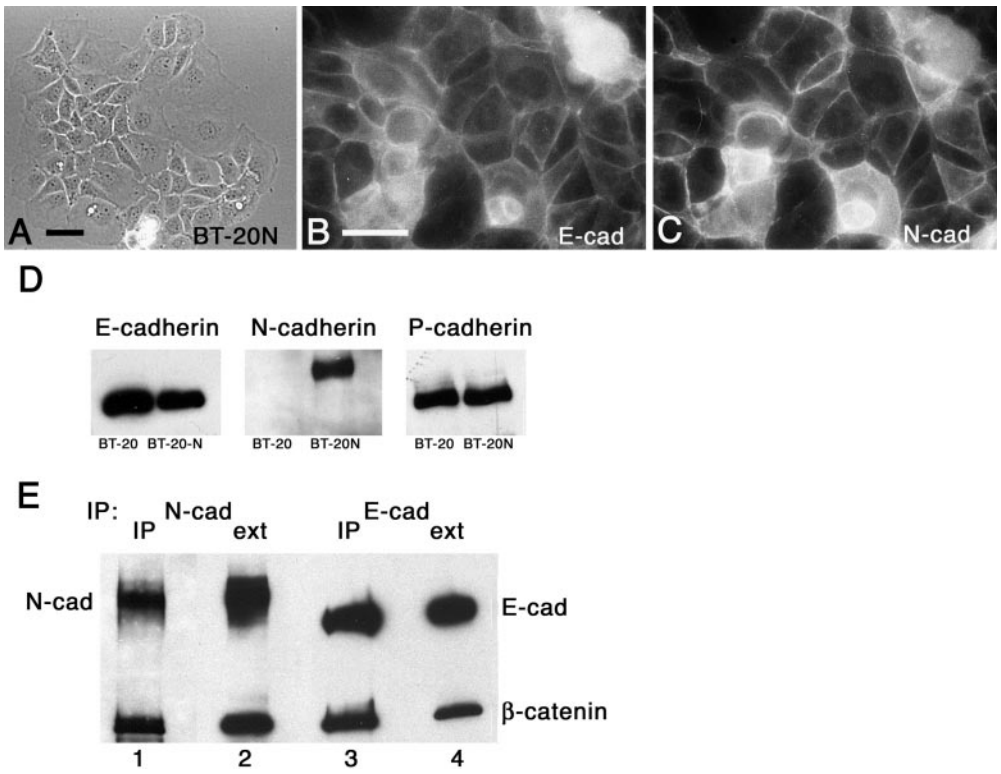

Figure 4. Expression of N-cadherin by BT-20 cells. BT-20 cells were transfected with N-cadherin (BT-20N) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20N cells. Bar, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and N-cadherin (C). D, BT-20 and BT-20N cells were extracted with NP-40 and 20 μg protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-cadherin (HECD1), N-cadherin, or P-cadherin. E, Extracts of BT-20N cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin (HECD1). The immunoprecipitation reactions, as well as cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β-catenin (lanes 1 and 2) or E-cadherin (HECD1) and β-catenin (lanes 3 and 4).

with the parental cells. Although these cells expressed very high levels of E-cadherin, they did not display a typical epithelial morphology, and closely resembled the parent cell line (compare Figs. 6 A with 2 F). Both E- and N-cadherin were localized to regions of cell–cell contact (Fig. 6, B and C). When the MDA-MB-435E cells were tested for motility and invasion, they were not significantly different from the parental MDA-MB-435 cells (Fig. 5), even though β-catenin was associated with the transfected E-cadherin, as well as the endogenous N-cadherin (Fig. 6 E).

BT-20N Cells Effectively Segregate from HT1080 Fibroblasts

Hazan et al. (1997) suggested that N-cadherin–expressing breast cancer cells invade the stroma because they associate with the N-cadherin–expressing stromal cells. In our studies, we employed an in vitro invasion assay in which the cells invade an extracellular matrix that does not include any stromal cells. Thus, we can make the important statement that, in our studies, N-cadherin actively promotes invasion and motility. In Hazan et al. (1997), the investigators showed that N-cadherin–expressing breast cancer cells coaggregated with N-cadherin–expressing fibroblast-like cells. Since it has been suggested that it is the entire complement of cadherins expressed by a cell that determines its ability to associate with other cells, and that even cells expressing different levels of the same cadherin can sort from one another (Steinberg and Takeichi, 1994), we sought to determine if the BT-20N cells that express N-, E-, and P-cadherin would segregate from an N-cadherin–expressing fibroblast cell line, HT1080. Equal numbers of BT-20 cells and HT1080 cells, or BT-20N cells and HT1080 cells, were mixed together and allowed to settle on glass coverslips. They were then prepared for immunofluorescence analysis using antibodies against E- or N-cadherin. In the immunofluorescence analysis of the BT-20/HT1080 cocultures, E-cadherin stained only the BT-20 cells and N-cadherin stained only the HT1080 cells. Fig. 7, A and B, show that these two cell lines effectively segregated from one another as expected. In the immunofluorescence analysis of the BT-20N/HT1080 cocultures, antibodies against E-cadherin stained only the BT-20N cells, whereas antibodies against N-cadherin stained both the BT-20N cells and the HT1080 cells. Fig. 7, C and D, show that the BT-20N cells and the HT1080 cells effectively segregated from one another, even though both cell lines express N-cadherin. Thus, epithelial cells that express N-cadherin along with other cadherins have not necessarily gained the ability to intermix with stromal cells.

Cadherin-11 Promotes Motility in Breast Epithelial Cells

In the course of our studies on breast tumor cell lines, we characterized one atypical line (MDA-MB-231) that did

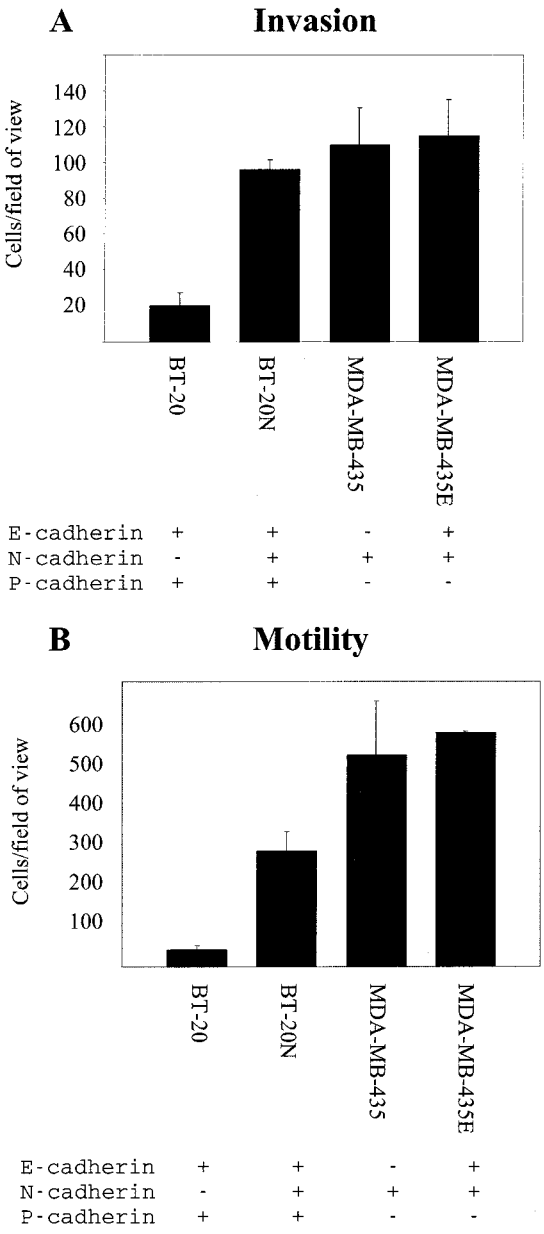

Figure 5. Exogenous expression of N-cadherin by BT-20 cells (BT-20N) increases their invasiveness, whereas exogenous expression of E-cadherin by MDA-MB-435 cells (MDA-MB-435E) does not effect their behavior. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

not express E-, P-, or N-cadherin, but nonetheless was invasive (Table I). Since MDA-MB-231 cells expressed significant levels of β-catenin, a protein that is not stable in cadherin-negative cells, we suspected that this cell line expressed another member of the cadherin family of proteins, possibly one that is closely related to N-cadherin. We therefore analyzed RNA from this line with degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin-11 mRNA. Expression of cadherin-11 protein was confirmed by immunoblotting data with a cadherin-11–specific mAb, in agreement with recent data (Pishvaian et al., 1999). Like N-cadherin, cadherin-11 is expressed by some mesenchymal cells (Simonneau et al., 1995). Interestingly, cadherin-11 is expressed in some epithelial cells of the human placenta, and it has been suggested that cadherin-11 plays a role in mediating trophoblast–endometrium interactions as the cytotrophoblasts invade the uterine wall (MacCalman et al., 1996). Thus, one idea is that cadherin-11 could act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells. To test this idea, we transfected cadherin-11 into BT-20 cells (BT-20Cad-11 cells). Like the BT-20N cells, BT-20Cad-11 cells retained the morphology of their parent line, even though they expressed high levels of cadherin-11 at cell–cell borders (Fig. 8, A–C). As predicted, cadherin-11–expressing BT-20 cells were more invasive and motile than the parental BT-20 cells (Fig. 8, D and E). Interestingly, the cadherin-11–expressing cells were not as invasive or motile as the N-cadherin–expressing cells. For example, the MDA-MB-231 cells were not as motile as the MDA-MB-435 cells (Figs. 5 and 8). More significantly, the BT-20 cells transfected with cadherin-11 did not become as motile as they did when transfected with N-cadherin. This may be due to differences between the two cadherins, or differences in expression levels of the transfected cadherins. It is reasonable to speculate that the level of expression of the inappropriate cadherin is relevant since the cell line SUM1315 expresses a small amount of cadherin-11, yet is not invasive.

N-Cadherin May Promote Cell Motility through a Fibroblast Growth Factor Receptor Signal Transduction Pathway

The laboratories of Frank Walsh and Patrick Doherty have shown that N-cadherin promotes neurite outgrowth from cerebellar neurons (Williams et al., 1994a). In addition, they showed that N-cadherin–mediated neurite extension was dependent on FGF receptor signaling, but was independent of ligand (Williams et al., 1994b). Walsh and Doherty thus proposed a model whereby the FGF receptor was induced to dimerize in the absence of FGF via interaction with N-cadherin (Doherty and Walsh, 1996). Dimerization of the FGF receptor results in receptor cross phosphorylation that initiates a number of signal transduction pathways. The pathway relevant to N-cadherin–dependent neurite outgrowth involves the generation of arachidonic acid from diacylglycerol, by the action of diacylglycerol lipase. The Walsh and Doherty laboratories showed that the diacylglycerol lipase inhibitor, RHC 80267, prevented neurite extension on N-cadherin–transfected 3T3 cells, thus implicating this type of FGF receptor signaling in N-cadherin–dependent neurite extension (Meiri et al., 1998). We hypothesized that the N-cadherin–mediated cell motility we observed in epithelial cells may also be acting through FGF receptor signaling. To test this hypothesis, we treated MDA-MB-435 cells, BT-20 cells,

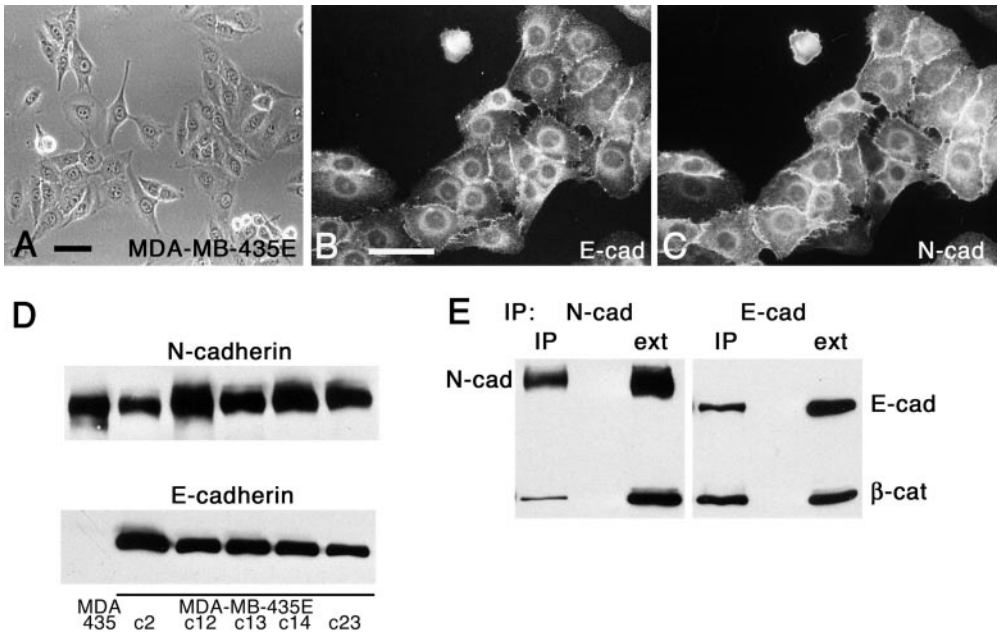

Figure 6. Expression of E-cadherin by MDA-MB-435 cells. MDA-MB-435 cells were transfected with E-cadherin (MDA-MB-435E) and expression was induced with dexamethasone. A, Phase-microscopy of MDA-MB-435E cells. Bar, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E- (Jelly; B) and N-cadherin (C). D, MDA-MB-435 and several clones of MDA-MB-435E cells were extracted with NP-40 and 20 μg protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E- (HECD1) and N-cadherin. Clone 2 (cl2) expressed the highest level of E-cadherin and was chosen for subsequent studies. E, Extracts of MDA-MB-435 and MDA-MB-435E cells were immunoprecipitated with antibodies against N- or E-cadherin (HECD1). The immunoprecipitation reactions, along with cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β-catenin (lanes 1 and 2), or E-cadherin (HECD1) and β-catenin (lanes 3 and 4).

and BT-20N cells with varying levels of RHC80267 to determine if it would influence the motility of these cells in the transwell assay. RHC80267 inhibited cell motility in both N-cadherin–expressing cell lines in a dose-dependent manner (Fig. 9 A). Importantly, this inhibitor had no effect on the motility of the N-cadherin–negative BT-20 cells. Although these data are consistent with the hypothesis that N-cadherin dependent cell motility is mediated through FGF receptor signaling in a manner similar to N-cadherin–dependent neurite outgrowth, additional experiments must be done to further support this notion. Thus, we are continuing to investigate the mechanism whereby N-cadherin mediates motility in epithelial cells. To determine if cadherin-11 and N-cadherin promote cell motility through a similar pathway, we treated MDA-MB-231 and BT-20cad11 cells with RHC80267, and compared

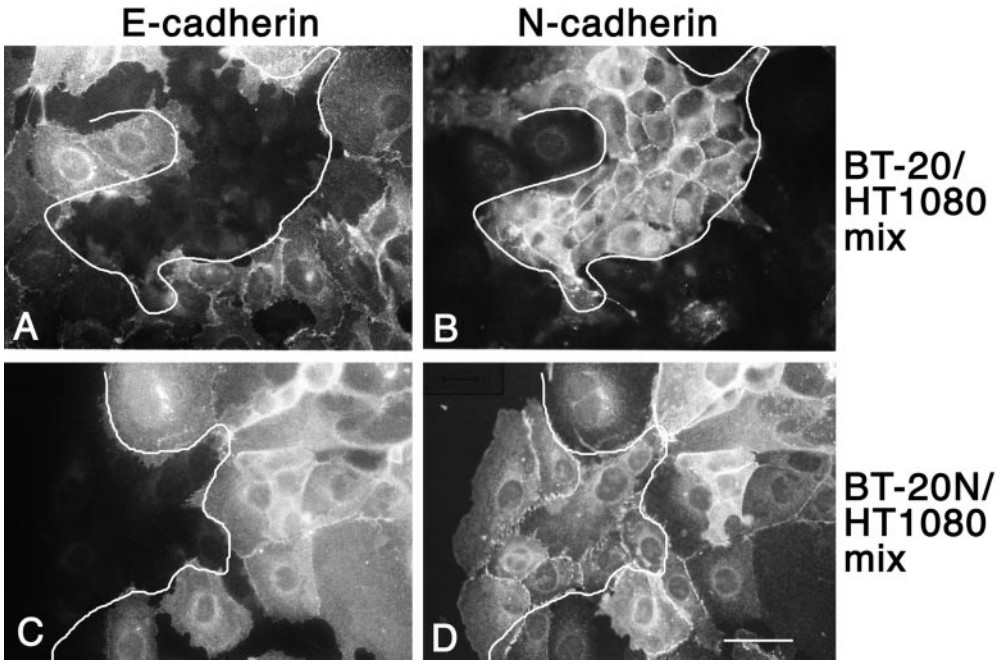

Figure 7. BT-20N cells do not mix with HT1080 cells. 5×10^4 BT-20 or BT-20N cells were mixed with an equal number of HT1080 cells, allowed to settle on coverslips, and processed for immunofluorescence with an mAb against N- (13A9) or E-cadherin (Jelly). A and B are a mix of BT-20 and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin–negative, N-cadherin–positive HT1080 cells. C and D are a mix of BT-20N and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin–negative, N-cadherin–positive HT1080 cells.

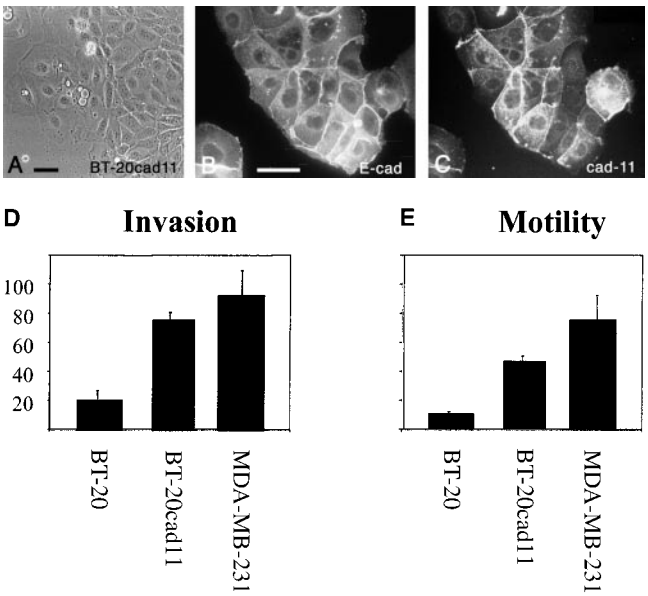

Figure 8. Exogenous expression of cadherin-11 by BT-20 cells (BT-20cad11) increases their invasiveness. BT-20 cells were transfected with cadherin-11 (BT-20cad11) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20cad11 cells. Bars, 10 μm. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and cadherin-11 (C). D and E, Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100 ×. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

motility rates between treated and nontreated cells (Fig. 9 B). The diacylglycerol lipase inhibitor decreased the motility of cadherin-11–expressing cells in a dose-dependent manner. Cadherin-11–expressing cells are less motile than MDA-MB-435, and the inhibitor is less effective in decreasing the motility of the cadherin-11 expressing cells, suggesting there may be some differences in the respective signal transduction pathways, possibly in growth factor receptor levels or isoforms.

Discussion

Previously, our laboratory showed that expression of different cadherin family members by squamous epithelial cells markedly effected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell–cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell–cell contacts, whereas breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin–expressing BT-20 breast cancer cell line had no effect on morphology, even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin–expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we characterized previously. Interestingly, the oral squamous epithelial cells downregulated E-cadherin when they were forced to express N-cadherin, suggesting an inverse relationship between these cadherins. In contrast, the breast cancer cells continued to express their endogenous cadherin(s) when transfected with a different cadherin. The continued expression of endogenous cadherin may account for the lack of morphological change in the transfectants. Thus, the breast cancer cells differ from the oral squamous epithelial cells in two very important ways: first, the oral squamous epithelial cells appear to coregulate cadherins in an inverse manner, whereas these cadherins are independently regulated in breast cancer cells; and second, expression of E-cadherin by the oral squamous epithelial cells is sufficient for epithelial morphology, whereas epithelial morphology in the breast cancer cells appears to depend on other factors, in addition to E-cadherin.

In the present study, we have demonstrated that N-cadherin (or cadherin-11) expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the BT-20 cells with these nonepithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin or cadherin-11 with invasion in breast cancer cells. However, in this study, we took the important next step and used transfection studies to show that a cell line that has a low invasion rate could be converted to a highly invasive cell by expression of N-cadherin or cadherin-11. The BT-20 breast cancer cell line provided an important tool for these studies since they did not downregulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin (or cadherin-11) can promote motility, suggesting that, in this regard, both N-cadherin and cadherin-11 are dominant over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin–negative breast cancer cell lines, BT549 and HS578, did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

A previous study using MDA-MB-435 cells showed that transfection of E-cadherin into these cells reduced their capacity to form tumors when injected into the foot pads of nude mice (Meiners et al., 1998). In contrast to our study, these authors showed that E-cadherin–transfected clones of MDA-MB-435 cells underwent a morphological

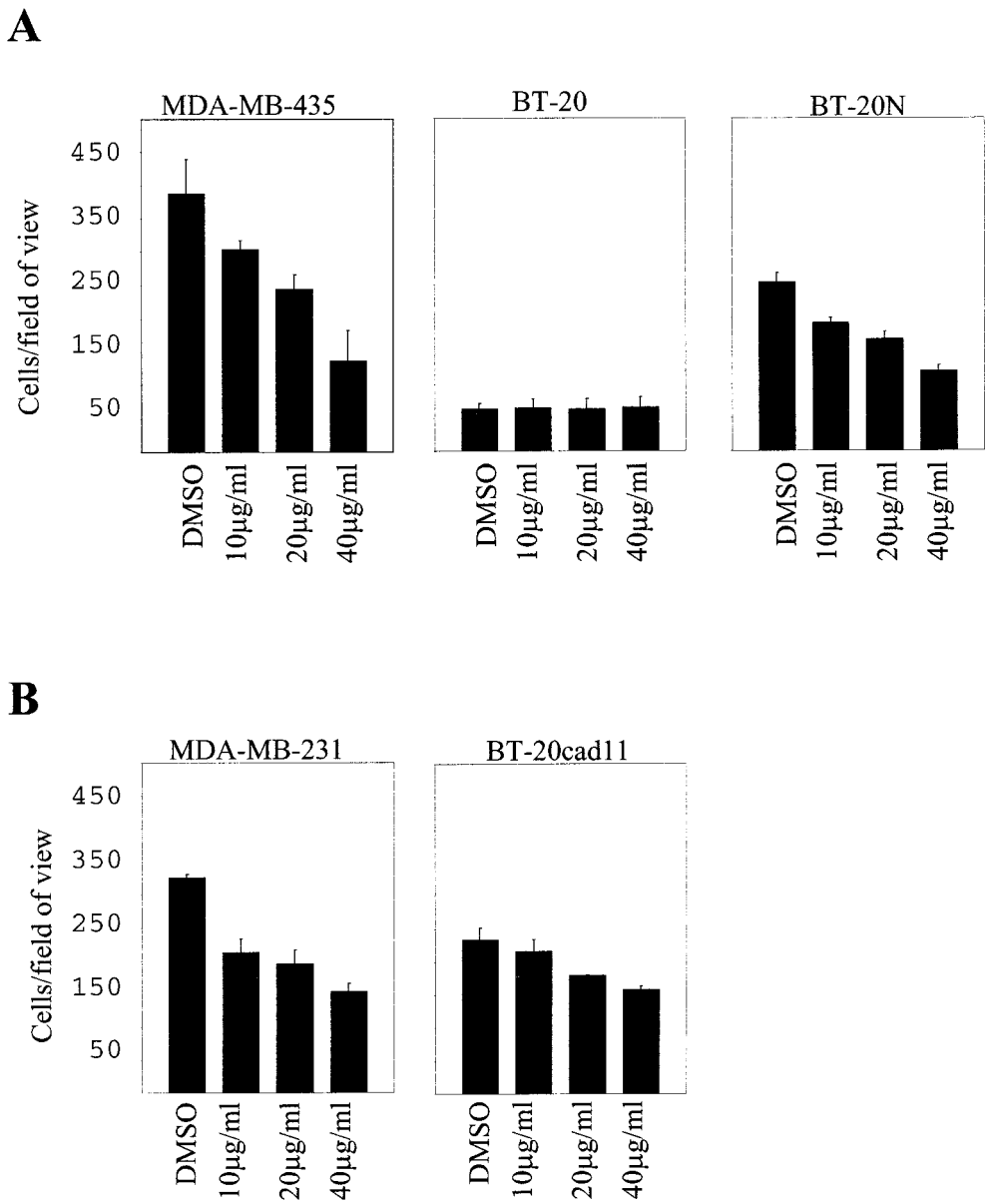

Figure 9. The diacylglycerol lipase inhibitor RHC80267 decreases motility of N-cadherin– and cadherin-11–expressing cells. Cells were plated on noncoated membranes for motility assays. The cells were incubated for 24 h in the presence of RHC80267 at varying concentrations, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transversing the membrane was determined by averaging ten random fields of view at 100×. The data are expressed as the number of cells/field of view and is the average of three (A) or two (B) independent experiments. Error bars indicate SD of the average.

change upon E-cadherin expression. In addition, they showed that E-cadherin–transfected clones were less tumorigenic in their assay than the parental cells. One difference in the study of Meiners et al. (1998) and ours is that they did not assay for N-cadherin expression in their E-cadherin–positive clones of MDA-MB-435 transfectants. Our study clearly demonstrates that N-cadherin influences the behavior of the cells, and that cells retaining N-cadherin do not undergo a morphological or behavioral change upon expression of E-cadherin. Thus, one possible explanation for the difference between these two studies is that the cells in the Meiners' study did not express N-cadherin. The point of our study was to determine if N-cadherin was capable of influencing the behavior of epithelial cells, even if they expressed E-cadherin, thus, we were particularly careful to select cell lines that retained N-cadherin expression after transfection with E-cadherin (Fig. 6).

One puzzling aspect of cell lines derived from metastatic tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by the present study is that such cells may have upregulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade, even though they continued to express E-cadherin. In this regard, expression of an inappropriate cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Some of the E-cadherin–negative breast cancer cells expressed endogenous P-cadherin. These cells had a fibroblastic morphology similar to that of the N-cadherin–expressing cells; however, they were not highly invasive, suggesting that P-cadherin confers upon breast cancer cells characteristics different from those conferred by either E- or N-cadherin. P-cadherin is expressed in the myo-

epithelial cells surrounding the lumenal epithelial cells of the mammary gland. Radice et al. (1997) recently showed that P-cadherin deficient mice develop age-dependent hyperplasia and dysplasia of the mammary epithelium, and suggested that P-cadherin may play a role in maintaining the normal phenotype of breast epithelial cells. One possibility is that the P-cadherin–expressing tumor cells were derived from the myoepithelium, rather than from the true epithelium.

E-cadherin has been termed an invasion suppressor because transfection of this protein into some E-cadherin–negative invasive carcinoma cells resulted in decreased invasive capacity. Our prediction is that at least some of these cell lines cells expressed a cadherin, like N-cadherin or cadherin-11, and overexpression of E-cadherin resulted in downregulation of the endogenous cadherin, as we saw with the oral squamous epithelial cells. Thus, we hypothesize that the invasion suppressor role of E-cadherin arises in part from its ability to decrease the level of N-cadherin in certain, but not all, tumors. In the present study, cell lines that did not express any classical cadherins, as evidenced by lack of β-catenin protein, as well as lack of detectable cadherin, had low invasion rates. Our hypothesis, that loss of E-cadherin alone does not necessarily increase invasive capacity in breast carcinoma cells, is supported by the observation that function-blocking antibodies against E-cadherin did not confer a highly motile, invasive phenotype on MCF-7 cells, a breast cancer cell line that is E-cadherin–positive and N-cadherin–negative (Sommers et al., 1991). The current study suggests that, in some carcinoma cells, expression of N-cadherin, or a similar cadherin such as cadherin-11, may actually be necessary for increased motility and invasion. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (Vos et al., 1997). We might suggest that a subsequent event would be activation of the expression of an inappropriate cadherin, such as N-cadherin or cadherin-11.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors, such as hepatocyte growth factor and members of the FGF, EGF, and TGF families (Blay and Brown, 1985; Vallés et al., 1990; Behrens et al., 1991; Geimer and Bade, 1991; Gherardi and Stoker, 1991; Rosen et al., 1991; Miettinen et al., 1994; Savagner et al., 1994, 1997). Walsh, Doherty, and coworkers have established, through extensive studies on FGF receptor and cell adhesion molecules, that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin, via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor, and subsequent activation of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty, and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin–expressing epithelial cells may result in increased motility, similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin–dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin–expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin–negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways. Continued efforts in our laboratory are aimed at further defining the signal transduction pathway(s) that mediate cadherin-dependent motility in epithelial cells.

At first glance, it might seem unlikely that expression of an additional cell adhesion molecule would confer a motile and invasive phenotype upon an epithelial cell. However, motile cells, such as fibroblasts and myoblasts, express N-cadherin (Knudsen et al., 1995; Huttenlocher et al., 1998) and a switch from E- to N-cadherin occurs in the chick embryo when epiblast cells ingress through the primitive streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986). Another interesting cadherin switch occurs during establishment of the human placenta, where fetal cytotrophoblast cells invade the vasculature of the uterus. During this invasive process, the cytotrophoblast cells downregulate the expression of E-cadherin and upregulate vascular/endothelial (VE) cadherin (Zhou et al., 1997). Thus, it is feasible to suggest that increased expression of a nonepithelial cell cadherin, such as N-cadherin, could increase the invasive potential of tumor cells. Ongoing studies in our laboratory are designed to determine how N-cadherin differs from E-cadherin in its ability to induce cell motility. We hypothesize that E-cadherin does not have the ability to interact with the relevant growth factor receptors, and we are preparing chimeric molecules between E- and N-cadherin to test this hypothesis.

An important message from the present studies is that cadherins may not function identically in different cell types. The fact that cadherins may act differently in different cell types is particularly evident when comparing the current study with earlier studies showing that mouse L cells or S180 fibroblasts attained an epithelial morphology when transfected with either E- or N-cadherin (Nagafuchi et al., 1987; Hatta et al., 1988; Matsuzaki et al., 1990). It will be important in future studies to consider the cellular makeup, as well as the complement of cadherin family members, when interpreting data on cellular morphology and behavior.

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